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DÉTERMINATION DES EFFETS DE LA QUANTITÉ ET DE LA QUALITÉ SPECTRALE DE LA LUMIÈRE NATURELLE SUR L'APOPTOSE CHEZ DES LARVES DE PERCHAUDE (*PERCA FLAVESCENS*)

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LISTE DES SYMBOLES ET ABRÉVIATIONS

ADN	Acide désoxyribonucléique
AIC	Critère d'information d'Akaike
Bax	Protéine X associée à Bcl-2
Bcl-2	Protéine 2 des cellules B des CLL et des lymphomes
CPD	Dimères de pyrimidine cyclobutane
ELISA	Essai immunologique d'enzymes liées
HBSS	Solution saline balancée de Hank
MAP kinases	Kinases activées par les mitogènes
p53	Suppresseur de tumeur 53
PARPs	Polymérases polyADP-ribose
PBS	Tampon phosphate salin
ROS	Espèces réactives de l'oxygène
RUV	Rayonnement ultraviolet
UV	Ultraviolet
UV-A	Ultraviolet A
UV-B	Ultraviolet B
UV-C	Ultraviolet C

RÉSUMÉ

L'amincissement de la couche d'ozone provoque l'augmentation des radiations ultraviolettes qui atteignent la surface de la Terre avec des conséquences potentielles pour les organismes, même dans les milieux aquatiques. Les UV-A (315 nm à 400 nm) et les UV-B (280 nm à 315 nm) sont reconnus pour causer la formation de dimères de pyrimidines dans l'ADN ainsi que la formation d'espèces réactives de l'oxygène, pouvant mener à un stress oxydant dans les cellules. Ces dommages peuvent mener une cellule à entrer en apoptose, un type de mort cellulaire programmée. Le but de mon projet de recherche a été de mesurer les effets du rayonnement ultraviolet sur l'apoptose, chez des larves de perchaude (*Perca flavescens*). Pour ce faire, les larves ont été exposées deux jours à la lumière solaire naturelle selon un plan expérimental factoriel croisant trois niveaux de qualité spectrale (lumière visible, lumière visible + UV-A ou lumière visible + UV-A + UV-B) et trois niveaux d'intensité lumineuse (forte, moyenne ou faible). Nous avons procédé à des immunobuvardages Western pour mesurer l'expression de certaines protéines apoptotiques et anti-apoptotiques telles que p53, Bax, Bcl-2 et PARP-1. Nous avons aussi mesuré la fragmentation de l'ADN, dernière étape de la cascade apoptotique. Enfin, nous avons fait un décompte des larves mortes pour obtenir le taux de survie aux différents traitements de lumière. Nos résultats montrent qu'après deux jours d'exposition aux traitements où les UV-A sont présents, l'expression des protéines Bax, p53 et Bcl-2 n'est pas différente du contrôle, mais que la fragmentation de l'ADN et l'expression de la protéine PARP-1 sont diminuées par rapport au contrôle. Cela suggère que l'apoptose n'emprunte pas la voie intrinsèque; des études ultérieures pourraient permettre de vérifier des marqueurs spécifiques à la voie extrinsèque. Comme la protéine PARP-1 varie de la même façon que la fragmentation de l'ADN, il semble qu'elle ne joue que son rôle de détecteur des dommages à l'ADN et qu'elle n'est pas réellement impliquée dans la cascade apoptotique. Nos résultats mettent également en

évidence que le rayonnement UV-A ralentit l'apoptose chez les larves de perchaude après deux jours d'exposition à différents traitements de lumière, suggérant que l'activation de la CPD photolyase par les UV-A est importante pour réparer les photo-dommages induits par la lumière solaire.

Mots clés : Larve de perchaude, UV-A, UV-B, photo-dommage, apoptose, photoréparation

CHAPITRE I

RÉSUMÉ SUBSTANTIEL

1.1 Introduction

1.1.1 Rayonnement ultraviolet (RUV)

1.1.1.1 Effets des RUV sur les organismes aquatiques

L'amincissement de la couche d'ozone dans la stratosphère provoque une augmentation de l'intensité des radiations ultraviolettes (UV) atteignant la surface de la Terre (Kerr et Mcelroy 1993). Les rayons UV les plus dommageables sont les UV-A (315 nm à 400 nm) et les UV-B (280 nm à 315 nm); les UV-C (200 nm à 280 nm) ne se rendent pas à la Terre, bien qu'ils soient les plus énergétiques (EPA 2010, Parisi et al. 2004). L'augmentation de ces radiations peut être nocive, autant chez les humains que chez les autres organismes vivants. Les écosystèmes aquatiques sont touchés par le rayonnement ultraviolet (RUV); les UV-B y pénètrent de plus en plus profondément à cause de l'amincissement de la couche d'ozone et de la diminution des matières en suspension dans l'eau (Häder et al. 2007). La modification de la transparence de l'eau, la quantité de carbone organique dissout, les pluies acides et l'eutrophisation contribuent également à moduler la quantité de RUV qui affecte les écosystèmes aquatiques (Frenette et al. 2006). Les rayons UV-B sont reconnus pour causer des dommages chez les organismes aquatiques (Williamson 1995). Une exposition à ce type de rayons peut causer des insolations, un ralentissement du taux de croissance et des lésions au cerveau et à la rétine chez les poissons (Hunter et al. 1979). Ces effets peuvent être évités par des stratégies défensives utilisées par les poissons, comme l'augmentation de pigments dans la peau (Häkkinen et al. 2002) ou la ponte des œufs plus en profondeur dans l'eau (Huff et al. 2004).

Pour certains organismes comme les larves de poissons, il est cependant difficile de se protéger contre le RUV. En effet, ce sont des organismes peu ou pas pigmentés, ils sont peu mobiles et ils ont un comportement de phototactisme positif (Jentoft et al. 2006, Mansueti 1964, Scott et Crossman 1974).

1.1.1.2 Effets des UV-B

Il a été rapporté que les larves de crapet arlequin (*Lepomis macrochirus*) et de morue de l'Atlantique (*Gadus morhua*) ont un taux de mortalité accru en présence de rayonnement UV-B (Béland et al. 1999, Olson et al. 2006). Plusieurs études ont aussi démontré que les UV-B provoquent la formation de dimères de pyrimidines (CPD) dans l'acide désoxyribonucléique (ADN) (Cadet et al. 2005, Malloy et al. 1997, Zeng et al. 2009). Ces dommages, lorsqu'ils sont trop importants, causent l'apoptose, un type de mort cellulaire, chez les larves de morue de l'Atlantique (Lesser et al. 2001). Le RUV peut déclencher la cascade apoptotique dans les cellules, menant à la fragmentation de l'ADN et l'élimination de la cellule (Gewies 2003). Cependant, l'apoptose n'est pas déclenchée que par des toxines ou des dommages à l'ADN. Par exemple, chez le poisson zébré (*Danio rerio*), l'apoptose est le mécanisme le plus utilisé dans le développement, permettant d'éliminer les cellules endommagées, non désirées ou mal situées (Eimon et Ashkenazi 2010, Greenwood et Gautier 2005, Penaloza et al. 2006). Il est donc normal d'observer une certaine quantité d'apoptose chez les organismes en développement telles les larves de poissons.

1.1.1.3 Effets des UV-A

Le rôle des UV-A est moins bien défini que celui des UV-B. Il a été démontré que, comme le font les UV-B, le rayonnement UV-A provoque la formation de CPD dans l'ADN (Douki et al. 2003, Rochette et al. 2003) et la

formation d'espèces réactives de l'oxygène (ROS) (Sage et al. 2005). Une trop grande quantité de ROS par rapport à la quantité d'antioxydants dans une cellule peut engendrer un stress oxydant, endommageant les protéines, les lipides et l'ADN (Monaghan 2009), menant éventuellement à l'apoptose. Par contre, il a aussi été démontré que les UV-A et la lumière visible jouent un rôle important dans la réparation de l'ADN par l'activation de l'enzyme CPD photolyase (Britt 1999, Friedberg 2003, Mitani et al. 1996). Il a été montré que la quantité de CPD augmente en avant-midi et diminue en après-midi en présence de lumière visible et d'UV-A, selon un cycle diurne (Vetter et al. 1999). Comme les UV-A sont autant reconnus pour les dommages qu'ils créent que pour la photoréparation qu'ils activent, il est difficile de définir clairement le rôle qu'ils jouent chez les organismes aquatiques. L'exposition aux UV induit donc à la fois des dommages directs à l'ADN et la formation de ROS, mais les dommages principalement étudiés chez les organismes aquatiques sont les CPD. On mesure également l'expression d'enzymes antioxydantes et de protéines de stress cellulaire comme l'enzyme superoxyde dismutase et la protéine p53 (Browman et al. 2003, Lesser et al. 2001). Les CPD et l'augmentation du stress oxydant sont deux facteurs qui peuvent mener une cellule à entrer en apoptose, puisque des dommages à l'ADN trop importants déclenchent la cascade apoptotique (Gewies 2003). Une étude a d'ailleurs démontré que les UV avaient pour effet d'augmenter l'apoptose chez les larves de poisson zébré (Yabu et al. 2001). Jusqu'à maintenant, la majorité des études portant sur les dommages dus aux UV chez les organismes aquatiques a été faite à l'aide de lampes UV. Quelques études portent sur le rayonnement ultraviolet naturel, mais l'apoptose n'y a pas encore été mesurée (Béland et al. 1999, Häkkinen et Oikari 2004).

1.1.2 Apoptose

1.1.2.1 Description

L'apoptose est un type de mort cellulaire important chez tous les organismes. C'est un processus biologique finement régulé, qui permet autant le développement, la morphogenèse, le maintien de l'homéostasie que l'élimination de cellules endommagées ou potentiellement dangereuses (Gewies 2003). Il s'agit d'une mort cellulaire programmée impliquant plusieurs facteurs pro- et anti-apoptotiques qui régulent une cascade d'activation complexe, menant éventuellement à la fragmentation de l'ADN de la cellule (Figure 1.1).

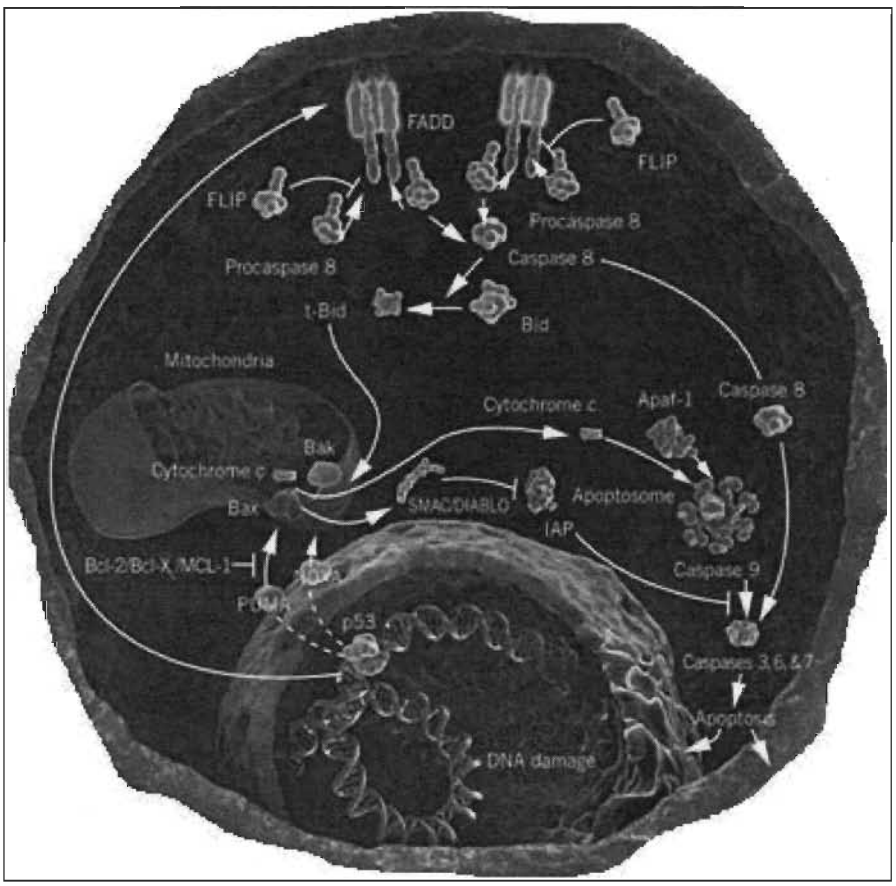

Figure 1.1 : Représentation schématique des voies intrinsèque et extrinsèque de l'apoptose (tiré de Genentech 2011).

Les cellules qui entrent en apoptose possèdent des caractéristiques bien précises : condensation de la chromatine, fragmentation de l'ADN, rétrécissement cytoplasmique, bourgeonnement de la membrane plasmique et finalement, phagocytose des débris par les macrophages. Ce type de mort cellulaire confère un avantage très important, contrairement à la nécrose par exemple, en empêchant l'inflammation, l'endommagement des tissus ou la mort des cellules avoisinantes (Zhang et al. 2004) (Figure 1.2).

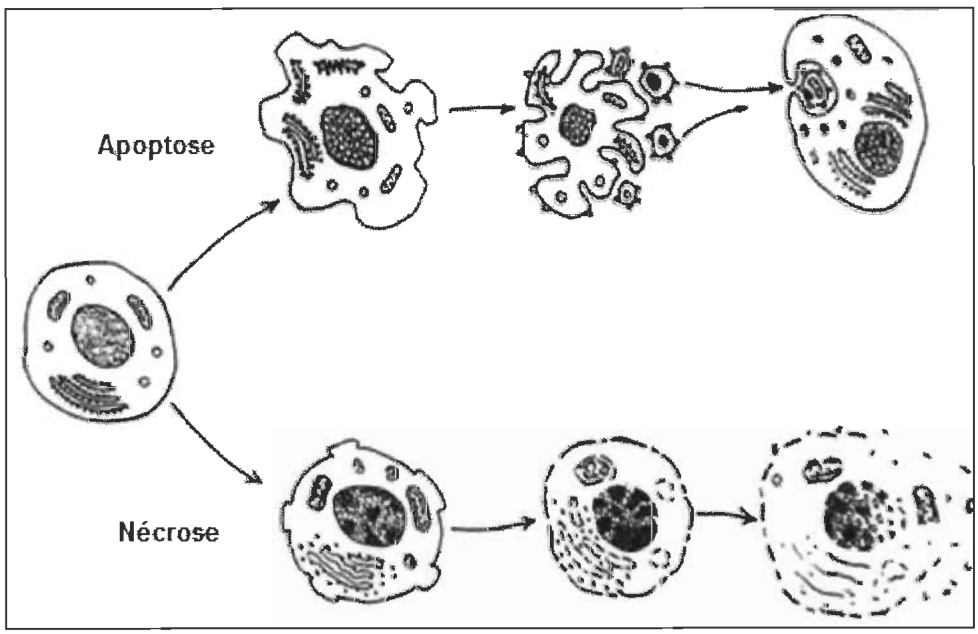

Figure 1.2 : Comparaison de deux types de morts cellulaires, l'apoptose et la nécrose (tiré de Meer et al. 2010).

L'apoptose peut être déclenchée par plusieurs facteurs tels les UV, les radiations ionisantes, le stress oxydant, des erreurs de réplication dans l'ADN ou encore des toxines (Batista et al. 2009, Nagata 1997, Norbury et Zhivotovsky 2004). L'apoptose peut agir comme un mécanisme de défense, par exemple dans une réaction immunitaire ou lorsque les cellules sont endommagées par des agents chimiques ou des maladies (Norbury et Hickson 2001, Plati et al. 2011). Le RUV est un facteur capable de déclencher la cascade apoptotique de plusieurs façons. Il peut enclencher la voie extrinsèque ou la voie intrinsèque, favoriser la formation de stress oxydant dans la cellule ou activer la voie des

MAP kinases (MAP kinases;*angl. :* "mitogen-activated protein kinases") (Batista et al. 2009). La voie extrinsèque consiste en un recrutement des complexes activateurs de l'apoptose suite à la liaison d'un facteur à un récepteur de la mort à l'extérieur de la cellule. La voie intrinsèque consiste en un déclenchement de la voie apoptotique suite à un signal provenant de l'intérieur de la cellule (Genentech 2011, Haupt et al. 2003).

1.1.2.2 Protéines pro-apoptotiques

Dans ce mémoire, nous nous sommes concentrés sur la voie intrinsèque, voie la plus souvent étudiée du phénomène d'apoptose. Les protéines pro-apoptotiques que nous avons étudiées sont p53, PARP-1 et Bax.

Le suppresseur de tumeurs 53 (p53) est une protéine impliquée dans la détection des dommages à l'ADN. Son principal rôle est d'arrêter le cycle cellulaire en cas de stress cellulaire trop important, d'où son rôle de suppresseur de tumeurs (Bartek et Lukas 2001, Latonen et Laiho 2005, Lodish 2005, Vousden et Lu 2002). Cependant, son activation peut également mener à la différenciation cellulaire, à la sénescence ou à l'apoptose (Vousden et Lu 2002). La protéine p53 est un facteur de transcription qui permet d'activer plusieurs gènes tels Bid, Noxa et Bax (Timares et al. 2008).

Les poly(ADP-ribose) polymérases (PARPs) sont des protéines qui catalysent une modification post-traductionnelle, la poly(ADP-ribosyl)ation. Cette modification consiste en l'ajout de polymères d'ADP-ribose à des protéines cibles (Ame et al. 2004, Bouchard et al. 2003). PARP-1 est le membre le plus abondant et le plus actif des PARPs (Bouchard et al. 2003, Cohen-Armon 2007). PARP-1 est rapidement activée par les bris simple-brin et double-brin et son auto-poly(ADP-ribosyl)ation permet le recrutement d'enzymes de réparation de l'ADN au site du dommage (Bouchard et al. 2003, de Murcia et Menissier de Murcia 1994, Mendoza-Alvarez et Alvarez-Gonzalez 1993). Une fois le

dommage réparé, PARP-1 quitte l'ADN, laissant un brin complètement réparé (Brochu et al. 1994, Miwa et al. 1974). Dans le cas d'un dommage à l'ADN trop important, PARP-1 mène la cellule à entrer en apoptose. À la toute fin de la cascade apoptotique, les caspases exécutives 3 et 7 clivent PARP-1 en deux fragments de 24 kDa et 89 kDa (Germain et al. 1999, Nicholson et Thornberry 1997). Lorsque les dommages à l'ADN sont limités, PARP-1 n'est pas impliquée dans la cascade apoptotique et n'est donc pas clivée (Ame et al. 2004).

La protéine X associée à Bcl-2 (Bax;*angl.* : "Bcl-2 associated X protein") est une protéine pro-apoptotique qui, sous forme de monomères, est transférée du cytoplasme vers la membrane mitochondriale suite à son activation par la protéine t-Bid (Gross et al. 1998). Une fois insérée dans la membrane externe de la mitochondrie, il y a oligomérisation de la protéine. Les homodimères de Bax provoquent un changement ionique dans l'espace intermembranaire, ce qui déclenche la sortie du cytochrome c dans le cytoplasme, entraînant l'apoptose (Jurgensmeier et al. 1998, Kluck et al. 1997, Lodish 2005, Reed 1997).

1.1.2.3 Protéine anti-apoptotique

L'unique protéine anti-apoptotique que nous avons étudiée dans le cadre de ce projet est la protéine 2 des cellules B des CLL et des lymphomes (Bcl-2;*angl.* : "B-cell lymphoma 2"). Il s'agit d'une protéine dont le rôle est de s'hétérodimériser avec Bax dans le cytoplasme pour le retenir (Lodish 2005, Oltvai et al. 1993). De cette façon, Bcl-2 empêche Bax de perméabiliser la membrane mitochondriale et bloque la cascade apoptotique. Nous avons mesuré l'expression de Bcl-2 pour pouvoir calculer le ratio d'expression des protéines Bax et Bcl-2 : c'est ce ratio qui indique s'il y a apoptose ou non dans une cellule (Bournival et al. 2009, Oltvai et al. 1993).

1.1.3 Organisme à l'étude

Le modèle d'étude utilisé pour ce projet a été la larve de perchaude (*Perca flavescens*) fraîchement éclose, au stade prolarvaire. La facilité d'échantillonnage des larves ainsi que la fragilité de ces organismes au rayonnement ultraviolet en ont fait un modèle d'étude idéal.

1.1.3.1 Fraye de la perchaude

Dans le sud du Québec, la fraie de la perchaude a lieu au printemps, habituellement du 15 avril jusqu'au début du mois de mai. Par contre, il arrive parfois que la période de fraie se prolonge jusqu'au mois de juillet, selon les régions et les conditions atmosphériques ambiantes. La perchaude est un poisson qui fraie la nuit ou encore très tôt le matin, moments où la température de l'eau varie entre 8,9 °C et 12 °C (Scott et Crossman 1974).

La fraie de la perchaude a lieu dans les hauts-fonds des cours d'eau ou dans la zone littorale, toujours près de la végétation. Les adultes migrent vers les eaux peu profondes des lacs, souvent vers les affluents (Canada 2010). La perchaude femelle libère un cordon transparent et gélatineux dans lequel sont contenus les œufs. Ce cordon a la forme d'un accordéon et adhère aux branches ou macrophytes présents au site de ponte. Lorsqu'il y a absence de végétation, la ponte peut aussi adhérer à des fonds rocailleux. La femelle laisse ce cordon sans surveillance pendant que les mâles viennent y déposer leur sperme (Scott et Crossman 1974). L'éclosion a lieu environ huit à dix jours après la fécondation et les larves restent inactives pendant près de cinq jours post-éclosion (Canada 2010).

1.1.3.2 Morphologie des larves de perchaude

Les larves fraîchement écloses sont au stade prolarvaire et leur longueur varie entre 1,7 mm et 3 mm. À ce stade, les nageoires ne sont pas encore bien

formées, les larves sont complètement transparentes, sans pigments et elles possèdent un sac vitellin, qui leur fournit l'énergie nécessaire au métabolisme pendant quelques jours après l'éclosion (Figure 1.3) (Canada 2010). À plus de 3 mm, la larve est toujours au stade postlarvaire et a les yeux pigmentés. À partir d'une longueur de 3,7 mm, il y a résorption du sac vitellin et l'apparition de pigments, soit des chromophores et des mélanophores (Mansueti 1964).

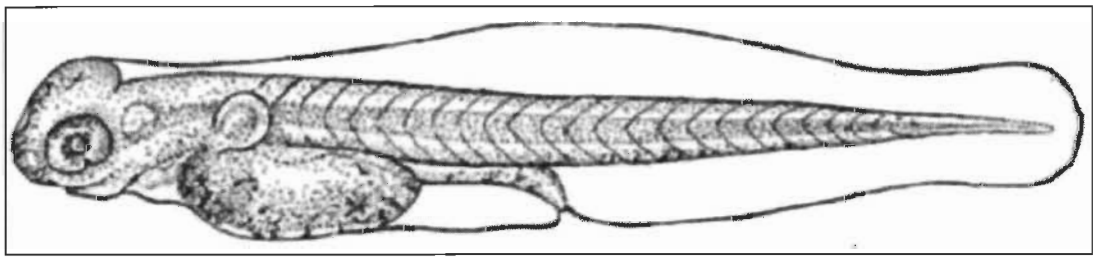

Figure 1.3 : Perchaude de 2 jours, au stade de prolarve tardive (tiré de Mansueti 1964).

1.1.4 Objectifs du projet de recherche

À notre connaissance, aucune étude n'a porté sur l'effet du RUV naturel sur l'apoptose chez les larves de poissons. Le but de cette étude a été de mesurer les effets de la qualité spectrale (UV-A ou UV-A + UV-B) et de l'intensité (forte, moyenne ou faible) du RUV naturel sur l'apoptose, chez des larves de perchaude (*Perca flavescens*). De façon plus spécifique, nous avons mesuré l'expression des protéines pro- et anti-apoptotiques Bax et Bcl-2 ainsi que des protéines p53 et PARP-1 suite à une exposition de deux jours, à neuf différents traitements de lumière, sous un rayonnement solaire naturel (plan factoriel 3x3 de l'intensité et du type d'UV). Nous avons également mesuré la fragmentation de l'ADN sur ces mêmes larves pour confirmer qu'il y avait bel et bien eu apoptose. Comme elle a été réalisée à l'aide de lumière solaire naturelle, notre étude permet d'obtenir des résultats plus représentatifs de l'impact des rayons UVA et UVB sur l'apoptose chez les organismes aquatiques (Williamson 1995).

1.2 Matériel et méthodes

1.2.1 Échantillonnage

Des œufs de perchaude ont été échantillonnés le 5 mai 2009 et le 15 avril 2010, à l'Île du Milieu, sur la rive nord de l'Archipel des îles de Sorel-Berthier, fleuve Saint-Laurent, Québec, Canada. Les pontes ont été incubées dans des aquariums du Laboratoire de Recherche sur les Communautés Aquatiques de l'Université du Québec à Trois-Rivières à des températures d'environ 18 °C à 20 °C. Les pontes ont été secouées afin de faire éclore tous les œufs en même temps, permettant ainsi l'obtention de larves au même stade de développement.

1.2.2 Exposition des larves au rayonnement solaire complet

Des groupes de 30 individus en 2009 et de 45 individus en 2010 ont été formés aléatoirement à l'aide de larves tout juste écloses. Ces groupes ont été placés dans des incubateurs faits à partir de contenants de plastique transparent avec un fond grillagé, empêchant les larves de sortir mais permettant une bonne circulation de l'eau. Un total de 40 incubateurs en 2009 et de 60 incubateurs en 2010 ont été installés dans des glacières de polystyrène, à raison de dix incubateurs par glacière. Les incubateurs ont été randomisés dans chacune des glacières, représentant l'effet bloc. L'effet d'ombrage des glacières n'a pas été tenu en compte. Un filtre mécanique fait de gravier, de sable et de laine de verre a été relié à chaque glacière pour assurer une filtration et une circulation adéquates de l'eau et un bloc réfrigérant a permis de stabiliser la température de l'eau. L'eau ajoutée dans chacune des glacières provenait de l'aqueduc municipal et a été décantée au moins 24 h avant la mise en place des larves afin de s'assurer que tout le chlore présent dans l'eau s'était évaporé.

Trois types de filtres sélectifs ont été utilisés pour moduler la qualité spectrale de lumière reçue : une pellicule plastique de type Whirlpak® (Nasco,

Atkinson, WI), qui laisse passer tout le RUV (ci-après appelée « lumière visible + UV-A + UV-B ») (Olson et al. 2006), une pellicule plastique de type Mylar-*d*® (SABIC polymershapes, Montréal, QC, Canada), opaque aux UV-B (ci-après appelée « lumière visible + UV-A ») et une pellicule plastique de type J-Roll® (SABIC polymershapes, Montréal, QC, Canada), opaque aux UV-A et aux UV-B (ci-après appelée « lumière visible » (Boily et al. 2011). La condition « lumière visible + UVB » n'a pu être obtenue faute de filtres disponibles.

Afin de moduler l'intensité de lumière reçue, des moustiquaires de fibres de verre servant de filtres neutres ont été installés sur les filtres sélectifs : les niveaux de traitement ont été constitués comme suit : aucune moustiquaire (ci-après appelée « intensité forte »), une couche de moustiquaire (ci-après appelée « intensité moyenne ») et deux couches de moustiquaire (ci-après appelée « intensité faible ») (Huggins et al. 2004). Trois niveaux de filtres sélectifs ont été croisés à trois niveaux de filtres neutres, ce qui a permis d'obtenir un plan factoriel complet (3 X 3).

Les larves ont été exposées au rayonnement solaire pendant trois jours en 2009 (13 au 15 mai, plein soleil-pluie-plein soleil) et pendant deux jours en 2010 (20 et 21 avril, deux jours de plein soleil), sur le campus de l'Université du Québec à Trois-Rivières. En 2009, un sous-échantillon de larves actives et un sous-échantillon de larves mortes ont été prélevés aux jours un et trois de l'expérimentation. En 2010, un sous-échantillon de larves mortes a été prélevé aux jours un et deux, alors qu'un sous-échantillon de larves actives et un sous-échantillon de larves inactives ont été prélevés au jour deux de l'expérimentation. Les larves dites mortes sont celles qui présentaient une morphologie caractéristique; gonflées et entourées de filaments. Les larves dites inactives sont celles qui ne présentaient pas la morphologie des larves mortes, mais qui ne nageaient plus. Finalement, les larves dites actives sont celles qui nageaient toujours dans les incubateurs au moment de les échantillonner. Les

larves retenues pour faire les expérimentations moléculaires sont les larves incubées en 2010 puisqu'en 2009 les larves n'ont pas été congelées de façon adéquate. Suite à ces prélèvements, les larves encore vivantes ont été euthanasiées dans de l'eau carbonatée et tous les échantillons ont été congelés dans du sucrose [30 % (p/v)] à -80 °C, de façon progressive pour éviter l'éclatement des cellules (glace, -20 °C, -80 °C). Les larves utilisées pour les expérimentations moléculaires sont les larves vivantes, puisque les larves inactives ou mortes présentaient des signes de dégradation physique, ce qui altérait la qualité des protéines et des cellules pour les expérimentations moléculaires. Aucune différence n'a été observée entre les traitements pour les larves mortes, actives ou inactives.

Pendant toute la durée de l'expérimentation, l'irradiance de surface a été mesurée avec un radiomètre PUV-2545/2546 (Biospherical Instruments Inc., San Diego, CA), placé sur le toit de l'Université du Québec à Trois-Rivières.

1.2.3 Détection des protéines apoptotiques et de stress cellulaire

Les protéines totales des larves ont été extraites à partir d'un mélange de trois larves par traitement selon la méthode d'extraction totale du Nuclear Extract Kit (Active Motif, Carlsbad, CA). Les protéines Bax, Bcl-2, p53 et PARP-1 a été quantifiée par la méthode d'immunobuvardage de type Western. Cette méthode semi-quantitative a pour but de mesurer la quantité de protéines cibles à l'aide d'anticorps primaires et secondaires. Après migration d'une quantité fixe de protéines sur un gel d'acrylamide, les protéines ont été transférées sur une membrane de nylon PVDF (pores de 0,2 µm, Bio-Rad, Hercules, CA). La membrane a ensuite été bloquée dans du lait en poudre afin que les protéines du lait adhèrent aux endroits de la membrane ne contenant pas de protéines de larves, évitant l'apparition d'un faux signal lors de la révélation. Les anticorps primaires polyclonaux utilisés ont été l'anti-Bax de lapin (1 :200, Delta Biolabs, Gilroy, CA), l'anti-Bcl-2 de lapin (1 :100, Santa Cruz

Biotechnology, Santa Cruz, CA), l'anti-p53 de lapin (1 :200, Santa Cruz Biotechnology, Santa Cruz, CA) et l'anti-PARP-1 de lapin (1 :50, Santa Cruz Biotechnology, Santa Cruz, CA). À la suite d'une incubation avec un anticorps primaire, des lavages ont été effectués dans du tampon phosphate salin (PBS;*angl. :* "phosphate buffer saline") puis l'anticorps secondaire de chèvre polyclonal anti-lapin, conjugué à l'enzyme peroxydase du raifort, a été incubé sur la membrane, permettant une meilleure spécificité du signal. Les signaux ont été détectés par chimiluminescence à l'aide d'un appareil AlphaEase FC (Alpha Innotech, San Leandro, CA) et analysés à l'aide du logiciel AlphaEase FC. Une technique d'inactivation de la peroxydase a été utilisée entre chaque anticorps primaire utilisé sur une même membrane (Sennepin et al. 2009). Après chaque détection chimiluminescente, les membranes ont été rincées à l'éthanol puis incubées dans du peroxyde d'hydrogène et rebloquées avant d'y remettre le prochain anticorps. Cette technique permet la réutilisation d'une même membrane et donc d'une faible quantité de protéines, pour mesurer toutes les protéines étudiées dans ce projet.

1.2.4 Détection des cellules apoptotiques par ELISA

Plusieurs techniques permettent la détection de cellules apoptotiques; dans ce cas-ci, une technique d'ELISA (*angl. :* "Enzyme-Linked ImmunoSorbent Assay") a permis de détecter et de quantifier l'apoptose chez les cellules de larves de perchaude exposées à différents traitements de lumière. Comme le test ELISA nécessite l'utilisation de cellules cultivées directement dans une plaque de 96 puits ou encore de suspensions cellulaires transférées par la suite dans une plaque de 96 puits, il a été nécessaire de faire des suspensions de cellules à partir des larves de perchaude entières (Freshney 1987).

Les larves ont été coupées en morceaux et ces derniers ont été rincés dans une solution de Hank (HBSS;*angl.* : "Hank's Balanced Salt Solution"). Une

solution sans calcium ni magnésium a été utilisée puisque ces ions interfèrent avec l'enzyme trypsine qui sera utilisée pour détacher les cellules du tissu (Invitrogen 2010). Une fois la solution HBSS enlevée, trois larves de chacune des conditions expérimentales ont été regroupées pour diluer l'effet individuel. Ces larves ont été incubées 6 h dans de la trypsine afin de maximiser la pénétration de l'enzyme dans les tissus. Après cette incubation, la trypsine a été retirée, puis les morceaux de tissus ont été incubés dans la trypsine résiduelle pour obtenir une activité enzymatique maximale. Par la suite, les tissus ont été resuspendus à l'aide d'une pipette dans du milieu de culture complet afin d'éviter l'éclatement des cellules. Cette suspension cellulaire a été filtrée à travers des tamis cellulaires de nylon stériles (pores de 100 µm; BD Biosciences, Mississauga, Ontario, Canada). Les cellules ont été comptées à l'aide d'un hématimètre dans du bleu Trypan.

La détection des cellules apoptotiques par ELISA a été faite selon le protocole décrit dans le ssDNA Apoptosis ELISA Kit (Chemicon International, Billerica, MA). Cette trousse permet de détecter spécifiquement les bouts d'ADN simple-brin dans les cellules apoptotiques grâce au formamide, un agent dénaturant l'ADN dans les cellules apoptotiques seulement (Frankfurt et Krishan 2001). La solution ABTS (*angl. :* 2,2'-AZINO-bis [3-éthylbenziazoline-6-sulfonic acid]) a été ajoutée pour permettre la détection par absorbance à une longueur d'onde de 405 nm sur un lecteur de plaques (Fisher Scientific, Ottawa, Ontario, Canada), après environ 30 min d'incubation, selon la coloration.

1.2.5 Analyses statistiques

Nous avons utilisé des modèles linéaires généralisés pour interpréter la réponse de nos variables dépendantes. Pour chaque variable étudiée, nous avons construit un modèle mixte prenant en compte l'effet bloc, ou facteur aléatoire, et les facteurs qualité, quantité, ainsi que leur interaction, ou facteurs fixes. Ces modèles ont été simplifiés par l'élimination des termes avec peu de

contribution à l'ajustement des modèles, à l'aide du critère d'information d'Akaike (AIC; Burnham and Anderson 2001). Les statistiques ont été réalisées à l'aide du logiciel R© (version 2.12.1, 2010).

1.3 Résultats

1.3.1 Transmittance des filtres

Les données de transmittance des filtres sont les mêmes que celles de Véronique Boily puisque les mêmes filtres ont été utilisés pour les expérimentations (Figure 1 dans Boily et al. 2011, annexe 3).

1.3.2 Taux de survie des larves exposées à différents traitements de lumière

Selon le Δ_i, le meilleur modèle pour expliquer le taux de survie des larves de perchaude exposées à différents traitements de lumière était le modèle D (Tableau 2.1a). Ce modèle inclut la quantité de lumière et explique moins de 1 % de la variation du taux de survie des larves. Ce taux de survie n'était pas différent d'un traitement à l'autre (Figure 2.1), et donc ni la qualité spectrale ni la quantité de RUV n'avaient d'effets significatifs sur le taux de survie après les deux jours d'exposition.

1.3.3 Effet des traitements de lumière sur l'expression des protéines pro- et anti-apoptotiques Bax, Bcl-2, p53 et PARP-1 chez des larves de perchaude

Le meilleur modèle pour expliquer l'expression de la protéine p53 était le modèle C (Tableau 2.1b), qui inclut la qualité spectrale de la lumière. Ce modèle explique 21 % de la variation de l'expression de p53. Le modèle D, qui inclut la quantité de lumière, explique la même fraction de l'expression de p53 que le modèle C. L'expression de p53 n'est pas différente d'un traitement à

l'autre (Figure 2.2). Ni la qualité spectrale ni l'intensité de la lumière n'ont eu d'effets sur l'expression de p53 pendant les deux jours d'exposition.

Pour mieux comprendre l'effet des UV sur la voie intrinsèque de l'apoptose, nous avons également mesuré le ratio d'expression des protéines Bax/Bcl-2, une mesure indirecte de l'apoptose fréquemment utilisée (Bournival et al. 2009, Li et al. 2010, Liu et al. 2010, Oltvai et al. 1993). Pour expliquer le ratio Bax/Bcl-2, les meilleurs modèles étaient les modèles C et D (Tableau 2.1c). Ces modèles incluent la qualité spectrale et la quantité de lumière reçue, et expliquent 2 % de la variabilité du ratio Bax/Bcl-2. Le ratio n'est affecté ni par la qualité ni par la quantité de RUV (Figure 2.3) après deux jours d'exposition au rayonnement solaire. Pour vérifier ce qui se passait plus loin dans la cascade apoptotique, nous avons également mesuré l'expression de la protéine PARP-1, impliquée à la fin de la cascade.

L'expression de PARP-1 était plus faible en présence des traitements lumière visible + UV-A et lumière visible + UV-A + UV-B, et plus élevée en présence du traitement lumière visible, sans être influencée par la quantité de lumière (Figure 2.4). Le meilleur modèle pour expliquer l'expression de PARP-1 inclut la qualité spectrale de la lumière (modèle C, tableau 2.1d). Ce modèle explique 30 % de la variabilité de l'expression de PARP-1.

1.3.4 Effet des traitements de lumière sur l'apoptose des larves de perchaude

Comme pour PARP-1, la fragmentation de l'ADN (i.e. l'apoptose), sans être influencée par la quantité de lumière, diminue en présence des traitements lumière visible + UV-A et lumière visible + UV-A + UV-B, par rapport au traitement lumière visible (Figure 2.5). Pour expliquer la fragmentation de l'ADN chez les larves de perchaude exposées à différents traitements de lumière, le meilleur modèle était le modèle C (Tableau 2.1e). Ce modèle explique 70 % de la variabilité de la fragmentation de l'ADN.

1.4 Discussion

Selon les résultats que nous avons obtenus, il semble que deux jours d'exposition au rayonnement solaire naturel n'aient pas été suffisants pour induire la mort des larves de perchaude. Comme l'ont montré Boily et al. (2011) dans des expériences similaires, le taux de survie des larves de perchaude exposées à la lumière visible + UV-A et à la lumière visible + UV-A + UV-B diminue après sept jours d'exposition au rayonnement solaire naturel. Il est donc possible que la mortalité des larves débute entre les jours deux et sept d'exposition.

L'expression du ratio Bax/Bcl-2 et de p53 n'a varié ni en fonction de la qualité spectrale ni en fonction de la quantité de lumière reçue. Normalement, ces deux marqueurs varient de la même façon que l'apoptose (Bournival et al. 2009, Lee et al. 2008, Lesser et al. 2001, Levine 1997, Oltvai et al. 1993). Comme il est bien connu que le RUV peut déclencher plusieurs voies de l'apoptose il semble que dans notre cas, l'apoptose ne soit pas déclenchée via la voie intrinsèque. Une autre explication possible serait le fait que dans plusieurs études portant sur l'apoptose induite par les UV, les expérimentations étaient réalisées quelques heures après l'incubation sous RUV (Kulms et al. 1999, Sitailo et al. 2002, Yabu et al. 2001), ce que nous n'avons pas fait ici étant donné que les larves ont été congelées immédiatement après les incubations.

Le fait que l'expression de PARP-1 varie de la même façon que l'apoptose suppose qu'elle n'est pas vraiment impliquée dans la cascade apoptotique. En effet, lorsque les dommages à l'ADN sont limités, PARP-1 détecte les dommages et permet le recrutement d'enzymes réparatrices de l'ADN (Brochu et al. 1994, Miwa et al. 1974). Une fois les dommages réparés, PARP-1 est libérée sans être clivée, ce qui a semblé être le cas dans nos expériences, comme si PARP-1 jouait son rôle de détecteur de dommages seulement.

Enfin, il semble que les UV-A et les UV-B sont capables de diminuer l'apoptose chez les larves de perchaude. La plupart des études montrent pourtant le contraire chez des embryons et larves de poissons (Browman et al. 2003, Lesser et al. 2001, Yabu et al. 2001). Il est possible que sous l'effet de la CPD photolyase, activée par les UV-A, les dommages à l'ADN soient diminués, ce qui entraînerait aussi une réduction de l'apoptose. Comme il a été mentionné dans l'introduction, il est normal d'observer un taux développemental d'apoptose chez un organisme. Une explication possible à nos résultats serait qu'en présence de RUV, l'apoptose est diminuée et que cela retarde le développement des larves de perchaude, causant éventuellement la mort des organismes.

En conclusion, nos résultats montrent que deux jours d'exposition ne sont pas suffisants pour induire une augmentation de l'apoptose chez des larves de perchaude. Par contre, en diminuant l'apoptose chez les larves, le RUV pourrait retarder le développement normal des larves. Nos résultats suggèrent également que les études faites à l'aide de lampes UV peuvent surestimer les effets du RUV sur l'apoptose et que des études plus poussées devraient être faites sur les effets du RUV naturel sur l'apoptose chez les larves de poissons.

1.5 Perspectives d'avenir

Dans une suite logique de ce projet, il serait intéressant d'étaler les expérimentations sur plusieurs jours afin de créer une cinétique dans le temps. Par exemple, les incubations pourraient être réalisées des jours un à sept. Cela permettrait de voir l'évolution de l'expression des protéines p53, Bax, Bcl-2 et PARP-1 ainsi que de la fragmentation de l'ADN et du taux de survie des larves. De plus, il serait important de mieux séparer les larves par état, soit actives, inactives ou mortes. De cette façon, il serait possible de voir la différence au niveau moléculaire entre chacun des états des larves. Finalement, il serait intéressant de refaire les incubations en laboratoire, à l'aide de lampes UV

mimant les doses naturelles; cette technique permettrait d'obtenir des expositions aux longueurs d'onde voulues, dans des conditions plus contrôlées qu'à l'extérieur. Ces expositions permettraient probablement d'obtenir des résultats plus clairs et plus faciles à interpréter.

CHAPITRE II

THE EFFECTS OF UVR IRRADIANCE AND SPECTRAL COMPOSITION ON APOPTOSIS IN YELLOW PERCH (*PERCA FLAVESCENS*) LARVAE

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Running head: UVR effects on apoptosis in yellow perch larvae

2.1 Résumé

Le but de cette étude était de déterminer expérimentalement les effets à court terme de la qualité (ratio UV-A/UV-B) et de la quantité (irradiance) du rayonnement ultraviolet (RUV) naturel sur les niveaux d'apoptose chez des larves de perchaude (*Perca flavescens*). L'apoptose a été mesurée par l'expression de protéines pro-apoptotiques (p53, Bax et PARP-1) et anti-apoptotiques (Bcl-2) et par la fragmentation de l'ADN. Des immunobuvardages de type Western ont été utilisés pour mesurer l'expression des protéines apoptotiques tandis que des essais ELISA ont permis de mesurer la fragmentation de l'ADN. Nous avions prédit qu'une exposition élevée au RUV serait reliée à des taux plus élevés d'apoptose. Nos résultats montrent que l'expression de p53 et que le ratio Bax/Bcl-2 n'étaient pas affectés de façon significative ni par la qualité ni par la quantité de RUV après deux jours d'exposition. La fragmentation de l'ADN ainsi que l'expression de PARP-1 étaient réduites par les traitements lumière visible + UV-A et lumière visible + UV-A + UV-B, mais n'étaient pas significativement affectées par la quantité de lumière. Nos résultats mettent en lumière le fait que le RUV peut réduire l'expression de marqueurs apoptotiques chez des larves de perchaude après deux jours d'exposition, ce qui suggère une implication spéciale du RUV dans la modulation de plusieurs paramètres développementaux de la vie aquatique.

2.2 Abstract

The aim of this study was to determine experimentally the short-term effects of the quality (UV-A/UV-B ratio) and quantity (irradiance) of natural ultraviolet radiation (UVR) on the apoptosis levels in Yellow perch (*Perca flavescens*) larvae. Apoptosis was measured by the expression of pro-apoptotic (p53, Bax, PARP-1) and anti-apoptotic proteins (Bcl-2), and DNA fragmentation. Western blots were used to measure the expression of the apoptotic proteins, whereas DNA fragmentation was measured by an ELISA assay. We predicted that higher UVR exposition will be related to higher levels of apoptosis and DNA

fragmentation. Our results show that the expression of p53 and the Bax/Bcl-2 ratio were not significantly affected in Yellow perch larvae by the quantity nor the quality of the light after 2 days of exposure. Whereas both DNA fragmentation and PARP-1 expression were reduced in visible light + UVA as well as in visible light + UVA + UVB treatments although they were not significantly affected by light quantity. All together our results enlighten that UVR may reduce apoptosis markers in Yellow perch larvae after two days of exposition, thus suggesting an intriguing involvement of UVR in the modulation of several developmental parameters of aquatic life.

Keywords: ultraviolet radiation, Yellow perch, fish larvae, apoptosis

Abbreviations: 2,2'-AZINO-bis [3-éthylbenziazoline-6-sulfonic acid] (ABTS), Akaike information criterion (AIC), B-cell CLL/lymphoma 2 protein (Bcl-2), Bcl-2-associated X protein (Bax), bovine serum albumin (BSA), cyclobutane pyrimidin dimers (CPD), deoxyribonucleic acid (DNA), horseradish peroxidase (HRP), phosphate buffer saline (PBS), poly (ADP) ribose polymerase 1 (PARP-1), polyvinyl difluoride (PVDF), reactive oxygen species (ROS), room temperature (RT), single strand DNA (ssDNA), sodium dodecyl sulfate (SDS), tris buffered saline-tween (TBS-T), ultraviolet A (UV-A), ultraviolet B (UV-B), ultraviolet C (UV-C), ultraviolet radiation (UVR).

2.3 Introduction

Ultraviolet radiation (UVR) is well known to cause damages in aquatic organisms (Williamson 1995), eventually leading to increased mortality (Bancroft et al. 2007). An exposure to UVR may result in sunburn, growth impairment and injuries to brain tissue and retina in fishes (Hunter *et al.* 1979). Most studies focussed on UV-B (280-315 nm), showing that these wavelengths can cause cyclobutane pyrimidine dimers (CPD) formation in deoxyribonucleic acid (DNA) (Cadet et al. 2005, Malloy et al. 1997, Zeng et al. 2009). Those damages may cause apoptotic cell death, as reported in studies on Atlantic cod

larvae exposed to UV-B (Lesser *et al.* 2001). Similarly to other stressors (i.e. toxins), UVR can initiate the apoptotic cascade through different pathways, all resulting in DNA fragmentation and cell removal (Gewies 2003; Batista *et al.* 2009). The two main apoptotic pathways, namely the intrinsic and extrinsic one, are respectively activated through an intracellular injury or by the activation of death receptors on the cellular membrane. On the other side, apoptosis is also the most important mechanism in pre- and post-natal development, allowing suppression of damaged, unwanted or badly located cells, as already reported by extensive works on zebrafish (*Danio rerio*) (Greenwood and Gautier 2005; Penaloza *et al.* 2006; Eimon and Ashkenazi 2010). Therefore, whereas a basal rate of apoptosis is normally observed in developing organisms such as fish larvae still going through morphogenesis, external stressors such as RUV may affect apoptosis by increasing its rates (Lesser et al. 2001).

The role of UV-A in inducing apoptosis is not as clear as for the UV-B, because while these wavelengths can cause photodammages to DNA, they can also induce DNA photoreparation (Douki et al. 2003, Mitani et al. 1996). Similarly to UV-B, UV-A may cause CPD formation in DNA (Douki *et al.* 2003; Rochette *et al.* 2003), and also induce reactive oxygen species (ROS) generation (Sage *et al.* 2005). A relatively high ROS/antioxidants ratio in the cell can lead to oxidative stress, which can damage proteins, lipids and DNA (Monaghan 2009), and eventually lead the cell towards apoptosis. On the other hand, UV-A and visible light may counteract these effects by activating the CPD photolyase enzyme (Mitani *et al.* 1996; Britt 1999; Friedberg 2003), which directly repairs CPD damages.

Most studies about UVR-induced apoptotic responses in aquatic organisms were performed under artificial light, with UVR lamps. These lamps are generally biased towards UV-B and do not adequately produce realistic UV-A/UV-B ratios (Häkkinen and Oikari 2004). In addition, in several studies reporting data obtained under natural UVR, apoptosis parameters were not

measured (Béland *et al.* 1999; Häkkinen and Oikari 2004, Boily et al. 2011). To our knowledge, nowadays, there are no data on the effect of natural UVR on apoptosis in fish larvae. Thus, the aim of this study was to measure the effects of spectral quality (visible light, visible light + UV-A or visible light + UV-A + UV-B) and intensity (high, medium or low) of natural UVR on several apoptosis markers in Yellow perch larvae (*Perca flavescens*). Indeed, we measured the expression of pro- and anti-apoptotic proteins, p53, Bax and Bcl-2, as well as the modulation of PARP-1 after two days of exposure to nine different natural solar light treatments (complete 3 X 3 experimental factorial plan). DNA fragmentation was also measured to validate cellular destruction. We predict that p53, the Bax/Bcl-2 ratio, PARP-1 and DNA fragmentation might be higher in the presence of both UV-B and UV-A, intermediate in the presence of UV-A only, and relatively low in the absence of UVR (Boily et al. 2011). We also predict that all these effect will increase along the light intensity gradient. Since our studies were performed under natural solar light, altogether our results allows a better perception of the impact of UVR on the balance underlying developmental apoptosis in aquatic organisms.

2.4 Material and methods

2.4.1 Material

All chemicals were purchased from Sigma (St-Louis, MO) unless specified otherwise.

2.4.2 Egg sampling

On April 15th 2010, Yellow perch eggs were sampled at Milieu's Island, on the north shore of Sorel-Berthier island archipelago, St.Lawrence River (Quebec, Canada) (46°04'52,06' N, 73°10'00.98' O). Eggs were incubated in aquariums of the Aquatic Communities Research Laboratory of Université du Québec à Trois-Rivières at temperatures between 18 °C and 20 °C. Egg strands

were gently shaked to help larvae hatching, to get larvae at the same development phase. After the reabsorption of the yolk sack, larvae were fed with Tetramin® flakes for young fishes.

2.4.3 Larvae exposition to natural solar light

Groups of 45 subjects were randomly formed with freshly hatched larvae. These groups were placed in incubators prepared with transparent plastic bowls (6 cm depth, 11 cm diameter) and with a ~200 µm mesh size net at the bottom. A total of 20 incubators were installed in two Styrofoam® coolers, each cooler containing ten incubators. Incubators were randomized in each cooler, the latter representing a "block".

Three different selective filters were used to modulate spectral quality of light: Whirlpak® (Nasco, Atkinson, WI) for full solar spectrum (hereafter called "visible light + UV-A + UV-B") (Figure 2.1 in Boily et al. 2011), Mylar-*d*® (SABIC polymershapes, Montréal, QC, Canada), opaque to UV-B (hereafter called "visible light + UV-A") and J-Roll® (SABIC polymershapes, Montréal, QC, Canada), opaque to UV-A and UV-B (hereafter called "visible light"). To modulate the intensity of light, three levels of neutral filters (window screens: none, one or two) were installed on the selective filters (Huggins *et al.* 2004). Three levels of wavelength selective filters were crossed with three levels of neutral filters to get a complete factorial plan (3 X 3). The control condition was obtained by covering incubators with an aluminium foil (hereafter called "dark"), which is useful to rule out whether the visible light treatment plays a role on apoptosis.

Larvae were exposed to complete solar spectrum for two days (April 20 and 21st 2010, two sunny days) on the Université du Québec à Trois-Rivières campus (Québec, Canada; 46°35' N, 72°58' O).

Samples of living larvae were taken at the end of the second day of the experiment. The larvae were euthanized with carbonated water, and all samples were frozen in 30 % (w/v) sucrose solution at -80 °C in a progressive way to prevent the breaking of cell membranes (ice, -20 °C, -80 °C).

During the experimental period, the surface irradiance was measured with a PUV-2545/2546 radiometer (Biospherical Instruments Inc., San Diego, CA) at a frequency of 5 Hz, at wavelengths of 313 nm and 320 nm for UV-B, 340 nm for UV-A and 400-700 nm for visible light. Percent transmission of each selective filter was calculated using the radiometer at the four wavelengths mentioned above from the ratio of the irradiance under the filter (measured with the radiometer covered with the different plastic films) and the total irradiance (measured with the uncovered radiometer) (Figure 2.1 in Boily et al. 2011).

2.4.4 Detection of apoptotic proteins

Total proteins were extracted from a mixture of three larvae according to the total extraction method from the Nuclear Extract Kit (Active Motif, Carlsbad, CA). The protein concentration was determined by using the BCA protein assay kit (Pierce Biotechnology inc., Rockford, IL). Expression of Bax, Bcl-2, p53 and PARP-1 proteins was quantified by Western blot as already reported (Bournival et al. 2009). Briefly, equal amounts of proteins were loaded on a 10 % SDS-polyacrylamid gel, and an electrophoretic separation was runned for 5 h at 120 V. The gels were transferred onto PVDF membranes (0.2 µm pores, Bio-Rad, Hercules, CA) and the blots were blocked for 1 h at room temperature (RT) in 5 % non-fat dry milk. Polyclonal primary antibodies used were: rabbit anti-Bax (1:200, Delta Biolabs, Gilroy, CA), rabbit anti-Bcl-2 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-p53 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-PARP-1 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA), all diluted in TBS-T with 0.5 g BSA and 25 mg

sodium azide. Blots were then incubated with HRP-conjugated polyclonal secondary antibody goat anti-rabbit (1:10,000) diluted in 5 % non-fat dry milk for 2 h at RT and developed with an enhanced chemiluminescence substrate solution (Haan and Behrmann 2007) with AlphaEase FC (Alpha Innotech, San Leandro, CA). After chemiluminescent detection, blots were rinsed in 100 % (v/v) ethanol, incubated for 15 min in 30 % (w/v) hydrogen peroxide at 37 °C and blocked for 1 h at RT in 5 % non-fat dry milk before addition of another primary antibody (Sennepin *et al.* 2009).

2.4.5 Detection of apoptotic cells by ELISA

Larvae were cut in two pieces with a sterilized scalpel to ensure a better penetration of the enzyme in tissues. Tissue pieces were rinsed in an Hank solution (with sodium bicarbonate) (Invitrogen, Burlington, Ontario, Canada) without calcium or magnesium. Then, three larvae for each condition were grouped to reduce the individual effect. Those three larvae were incubated for 6 h at 4 °C and for 20 min at 37 °C in 0.25 % trypsin (Invitrogen 2011).

Tissue pieces were crushed in RPMI 1640 medium supplemented with 10 % heat-inactivated horse serum and 5 % heat-inactivated fetal bovine serum. Cell suspension was filtered through a 100 µm pores nylon cell sieve (BD Biosciences, Mississauga, Ontario, Canada).

DNA fragmentation was detected using the ssDNA Apoptosis ELISA kit (Chemicon International, Billerica, MA), as reported (Bournival et al. 2009). This kit detects exclusively simple-strand DNA in apoptotic cells. Formamide is a selective denaturant for DNA in apoptotic cells, but not in necrotic cells or in DNA-damaged cells without apoptosis (Frankfurt and Krishan 2001).

Right before the test, about 8,000 cells per well were placed in a 96 wells plate and then harvested for 5 min at 300 g. Culture medium was removed and

the plate was incubated for 30 min at RT in 200 µl of fixative solution (80 % (v/v) methanol in PBS). Fixative was removed and the plate was dried overnight at RT. A second plate containing 100 µl of positive control was placed overnight at 37 °C. The following morning, plates containing experimental cells as well as control cells were incubated for 10 min in 50 µl of formamide at RT, following by incubations of 20 min at 75 °C and 5 min at 4 °C. Then, 100 µl of S1 nuclease (Invitrogen, Burlington, Ontario, Canada) were added to a negative control well and incubated for 30 min at 37 °C. From this step on, the two plates are treated in the same manner: three washes of 5 min with PBS and 1 h incubation at 37 °C in 3 % (w/v) non-fat dry milk in distilled water. Milk was removed and the plates were incubated in 100 µl of antibody solution for 30 min at RT. The antibody solution is a mix of primary and HRP-coupled secondary antibodies; just one incubation period is needed and the received signal is specific (Frankfurt and Krishan 2001). Three washes of 5 min were completed with Washing Solution from the kit, and the ABTS (2,2'-AZINO-bis [3-éthylbenziazoline-6-sulfonic acid]) was added. A specific signal was detect by absorbance at 405 nm wavelength, on a microplate reader (Fisher Scientific, Ottawa, Ontario, Canada) after about 30 min of incubation.

2.4.6 Statistical analysis

We used a general linear mixed modelling approach to compare the effects of light quantity and quality on Yellow perch larval apoptosis and survival. For each variable studied, we built a mixed model containing all variables that could predict the variable value: light quality and intensity (fixed effects), interaction between light quality and intensity (fixed effect) and block effect (random effect). Survival data (%) were arcsin transformed before their integration to the models. We used an information-theoretic framework to compare and rank the models. The Akaike Information Criterion (AIC) is selecting the best model to explain variation with the fewest variables. All models are ranked using $\Delta_i = AIC_i - AIC_{min}$, where AIC_i is the AIC value for a given model and AIC_{min} is the smallest

AIC value for the compared models. When $\Delta_i \leq 2$, a model has an important support, when $4 \leq \Delta_i \leq 7$ the support is considerable and when $\Delta_i \geq 10$ the model has less support. We used the AIC corrected for small size samples (AIC_c) for model selection because the ratio of the sample size and the number of parameters in the model is lower than 40 (Burnham and Anderson 2002). Model selection was based on the procedure suggested by Zuur et al. (2009). Briefly, we first compared the fit of two models containing all the fixed factors but differing by the presence of the random term. Restricted maximum likelihood estimation (REML) methods were used at this stage to determine if the random factor should be included in the model (Zuur et al. 2009). Second, we tested the contribution of the fixed factors to the fit of the model, by comparing models differing only in their fixed part. Maximum likelihood estimation (ML) methods were used at this stage (Zuur et al. 2009). At the end of the process, the selected model was refitted using REML. If the selected model included a random factor, we calculated the percent of variation explained by each model by squaring the Pearson correlation between observed values and values predicted by the full model (Deschênes and Rodriguez 2007). Otherwise, adjusted R^2 were calculated by refitting the data with a linear model. Statistical analysis were made with the functions lme(nlme), gls(nlme) and lm(stat) in the R© language (version 2.12.1, 2010).

2.5 Results

2.5.1 Transmittance of filters

The average percentages of visible light, UVA and UVB that passed through Whirlpak® filter (visible light + UVA + UVB) are 73.36 % ± 4.36 % (mean ± SD) for visible light (wavelengths between 400 nm and 700 nm), 59.19 % ± 2.96 % for UVA (wavelengths between 316 nm and 400 nm) and 50.04 % ± 1.93 % for UVB (wavelengths between 280 nm and 315 nm). Mylar-*d*® (visible light + UVA) had a mean transmittance of 88.06 % ± 0.98 % for visible light, 76.71 % ± 10.26 % for UVA and 1.34 % ± 4.12 % for UVB. The J-Roll® (visible

light) filter had a mean transmittance of 86.58 % ± 4.27 % for visible light, 6.30 % ± 11.17 % for UVA and of 0.11 % ± 0.04 % for UVB (Figure 2.1 in Boily et al. 2011). For one level of neutral density filter, 38.4 % ± 0.47 % of total irradiance was blocked and two levels of neutral density filter blocked 66.23 % ± 0.56 % of total irradiance.

2.5.2 Effects of UVR irradiance and spectral composition on Yellow perch larvae

The random factor was not included in any of the models since, for all the variables measured, the AICc suggested that its inclusion did not improve their fit.

2.5.3 Survival rate

According to the Δ_i, the best model to explain the yellow perch larvae survival rate includes only the light intensity (model D in table 1a). Besides being the best one, this model explains virtually no variation in larvae survival ($F_{2,15}$=0.46, P=0.630, R^2_{adj}<0.01), suggesting that neither spectral quality nor intensity of UVR had a significant effect on the mortality of larvae during the two first days of exposure (Figure 2. 1).

2.5.4 Expression of pro- and anti-apoptotic proteins Bax, Bcl-2, p53 and PARP-1

The best model to explain the p53 expression included only spectral composition (model C in table 1b) and explained virtually no variation in p53 protein expression ($F_{2,15}$=0.48, P=0.630, R^2_{adj}<0.01). The model including only light intensity (model D in table 1b) had virtually the same fit (Δ_i <2; $F_{2,15}$=0.12, P=0.890, R^2_{adj}<0.01). Therefore, neither spectral quality nor intensity affected p53 expression during the two days of exposition (Figure 2.2).

As for the p53 expression, the variation in the Bax/Bcl-2 ratio was best modeled by two models with similar fit (models C and D in table 1c). The models included either spectral quality or light quantity and explained virtually no variation in the ratio of Bax and Bcl-2 ($F_{2,15}$=1.29, P=0.303, R^2_{adj}<0.01, and $F_{2,15}$=0.57, P=0.580, R^2_{adj}<0.01, respectively). Therefore, this variable is not affected by spectral quality nor intensity of UVR (Figure 2.3) after two days of solar exposure.

The PARP-1 expression was lowest under visible light + UV-A and under visible light + UV-A + UV-B and highest under visible light treatment, whatever the light intensity (Figure 2.4). The best model to explain PARP-1 expression includes only spectral quality (model C, table 1d) and explains a non negligible fraction of the variation in the PARP-1 expression ($F_{2,15}$=6.155, P=0.0112, R^2_{adj}=0.38).

2.5.5 Apoptosis

As for the PARP-1 expression, the DNA fragmentation (i.e. the final step of the apoptotic cascade), was lowest under visible light + UV-A + UV-B and under visible light + UV-A and highest under visible light treatment, whatever the light intensity (Figure 2.5). The best model to explain the DNA fragmentation in Yellow perch larvae exposed to different light treatments was the one including only light spectral quality (model C in table 1e). This model explains a considerable fraction of the variation in DNA fragmentation ($F_{2,15}$=13.55, P<0.001, R^2_{adj}=0.596).

2.6 Discussion

Our results show that the UV-A and UV-B parts of the UVR can reduce the apoptosis rate as well as PARP-1 expression in Yellow perch larvae, while the expression of p53 and the Bax/Bcl-2 ratio are not affected.

According to our results, obtained under different levels of spectral quality and light intensity, UVR have no effects on larvae survival after two days of exposition. Whereas the expression of both p53 and the Bax/Bcl-2 ratio followed a similar pattern (i.e. lack of response to UVR), both PARP-1 and the DNA fragmentation showed clear, albeit unexpected, responses, being affected by the spectral composition treatments.

Under conditions similar to ours (i.e. same experimental setup, location and time of the year) but after a longer exposition (i.e. seven days), Boily *et al.* (2011) showed that UV-A and UV-B can have a strong negative effect on Yellow perch larvae survival. Other studies showed that UVR have a negative effect on larvae survival (Béland et al. 1999, Olson et al. 2006), but the mortality rate was measured following four to ten days of UVR exposition. Thus, it may be possible that two days exposure is not sufficient to affect the larvae survival.

Moreover, the response of both PARP-1 and DNA fragmentation was in fact opposite to our predictions: because UVR is known to induce apoptosis in culture cells (Bivik et al. 2006, Sitailo et al. 2002, Timares et al. 2008), we were expecting that UV-A and UV-B would increase DNA fragmentation and PARP-1 expression. On the contrary, we detected a decrease of these two apoptotic parameters and no variations of p53 and Bax/Bcl-2 ratio. Bcl-2 is a key member of the anti-apoptotic Bcl-2 family and is a potent inhibitor of apoptotic cell death (Cory et Adams 2002). Bcl-2 and Bcl-xL preserve mitochondrial integrity and prevent the subsequent release of apoptogenic molecules such as cytochrome *c* (Gollapudi et al. 2003). On the other hand, Bax is also a member of the Bcl-2 protein family. It accelerates the rate of apoptosis by a contribution to the permeabilization of the outer mitochondrial membrane, either by forming channels by themselves or by interacting with components of the outer mitochondrial membrane pore such as VDAC (Tsujimoto et Shimizu 2000). In this study, we examined in details the modulation of the gene expression of Bcl-2 and Bax after exposure to UVR. At the protein level, rise in the ratio of Bax to Bcl-2 indicates cellular improvement to apoptosis (Korsmeyer et al. 1993).

Our data reveal that Bax/Bcl-2 protein ratio is not modulated following exposure to UVR. Taken together, these results suggest that apoptosis is indeed affected by UVR, despite the fact that the exposure to natural light was possibly too short to detect any effect on larvae survival.

The exposition to UVR did not affect the expression of p53 and of Bax/Bcl-2 ratio. These apoptotic markers are considered as specific indicators of the intrinsic pathway of apoptosis and usually, their rising levels enhance the final step of apoptosis, i.e. DNA fragmentation (Lesser *et al.* 2001; Lee *et al.* 2008; Bournival *et al.* 2009). This result might be due to the prevalence of another apoptotic pathway not involving these proteins. This hypothesis is supported by several studies revealing that different apoptotic pathways may be initiated by UVR exposition (Lesser and Barry 2003; Hildesheim and Fornace 2004; Batista *et al.* 2009; Dahms and Lee 2010). As UVR can initiate the apoptotic pathway through the intrinsic and the extrinsic, as well as the mitogen-activated protein kinases or the ROS pathways, it is possible than one of those is induced faster than the intrinsic pathway. Actually, many data about UV-induced apoptosis have used protocols in which they wait for some hours after the UV incubation before the molecular experiments. This delay allows the cell to produce pro- and anti-apoptotic proteins (Kulms *et al.* 1999; Yabu *et al.* 2001; Sitailo *et al.* 2002). On the other hand, our protocol detect in an "in situ" situation, the possible modulation of the intrinsic apoptotic pathway.

Our results demonstrate that the expression of the PARP-1 protein is reduced in presence UV-A and of UV-A + UV-B. PARP-1 plays two different roles in the cell nucleus, as shown in several studies (Germain et al. 1999, Brochu et al. 1994). First, in the presence of DNA damage, PARP-1 detect the single-strands breaks and directly binds to them (Bouchard et al. 2003, de Murcia et Menissier de Murcia 1994, Mendoza-Alvarez et Alvarez-Gonzalez 1993) to recruit DNA repair enzymes to the damaged site. This leads to a complete repair of DNA strands (Miwa *et al.* 1974; Brochu *et al.* 1994). PARP-1 is also involved at the end of the apoptosis pathway: the executive caspases 3

and 7 cleaves PARP-1 in two fragments of 24 kDa and 89 kDa (Nicholson and Thornberry 1997; Germain *et al.* 1999). Once the protein is cleaved, enzymes like CAD (Caspase-Activated DNase) can proceed to the fragmentation of the DNA strands (Zhang and Xu 2000). Because PARP-1 is cleaved just before the DNA fragmentation, the entire PARP-1 quantity should decrease during the apoptotic process. However, our results show that the DNA fragmentation, after two days of exposition under different light treatments, varies in the same way than entire PARP-1 protein expression, thus suggesting that in our experimental paradigm PARP-1 plays its role of DNA damage detector, without being involved in the apoptotic pathway. Indeed, when the DNA damages are limited, PARP-1 activates the DNA reparation and is released once the DNA is completely repaired, without being cleaved, as sustained in our experiments. As the two treatments (visible light + UV-A and visible light + UV-A + UV-B) contain the UV-A part of the solar spectrum, it is possible that UV-A activates the enzyme CPD photolyase. This enzyme binds to a CPD in DNA strain, is directly activated by the 350-450 nm wavelengths and breaks the links between the two pyrimidines, without damaging the cell (Britt 1999; Friedberg 2003). Generally, less CPD in the DNA of a cell is linked to a decrease in apoptosis; as the yellow perch larvae are organisms in development, an important decrease of apoptosis due to CPD photolyase activation may be harmful to larvae. Those results are in agreement with the results showed in Boily et al. (2011), all showing that UV-A have a negative effect on larvae.

Our results clearly show that UV-A and UV-B reduce DNA fragmentation in Yellow perch larvae exposed to natural light. In contrast, most studies on effects of UVR in aquatic organisms showed that UVR, mostly UV-B, are responsible for the formation of CPD in DNA, eventually leading to the fragmentation of the DNA (Vetter *et al.* 1999; Lesser and Barry 2003; Karentz *et al.* 2004). Many studies also showed that UVR activates the apoptosis pathway, leading to DNA fragmentation in fish larvae and embryos (Lesser *et al.* 2001; Yabu *et al.* 2001; Browman *et al.* 2003). In this study, the apoptosis (i.e. DNA fragmentation) in

the visible light condition (Figure 2.5) is higher than in treatments where UVR is present. This could be explained by the fact that CPD photolyase, activated by UV-A, reduces the DNA damages and then decreases the apoptosis in Yellow perch larvae. The organisms in development, such as fish larvae, have a normal rate of apoptosis since this mechanisms is a crucial step in their morphogenesis (Eimon and Ashkenazi 2010, Greenwood et Gautier 2005, Penaloza et al. 2006). By showing that UVR are related to lower apoptosis levels, our results suggest that the exposure to these wavelengths act indeed on the apoptotic process by impairing morphogenesis in Yellow perch larvae.

In conclusion, whereas our results show that two days of exposition to UVR are not sufficient to cause the death of Yellow perch larvae, they clearly show that these wavelengths can affect the apoptotic cascade at cellular level. By showing that the apoptosis can be decreased rather than increased by exposure to UVR, our results open new pathways to the understanding of this stressor on living organisms in development. Understanding whether developing organisms are affected by UVR (increased stress or impaired morphogenesis) or they can set on their metabolic machinery to reduce UVR damages (DNA photorepair induced by UV-A), might be a key step to predict the outcome of UVR exposure in living organisms. It is now important to address more explicitly these questions by measuring simultaneously the photorepair and the apoptotic response in organisms exposed to UVR during their morphogenesis.

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2.9 Tables

Table 2.1

Set of candidate models to explain survival rate, p53 expression, Bax/Bcl-2 ratio, PARP-1 expression and DNA fragmentation of Yellow perch larvae. The selection of the model (in bold) was based on the Akaike information criterion corrected for small samples (AIC_c; see text for more details). All the comparisons are based on maximum likelihood estimates. a) survival rate; b) p53 expression; c) Bax/Bcl-2 ratio; d) PARP-1 expression; e) DNA fragmentation. μ = intercept, x1 = spectral quality, x2 = light intensity.

(a)			
Model		AIC_c	Δ_i
A	$y = \mu + \alpha x_1 + \beta x_2 + \varphi x_1 x_2$	--	
B	$y = \mu + \alpha x_1 + \beta x_2$	496.6	5.9
C	$y = \mu + \alpha x_1$	493.3	2.6
D	$y = \mu + \beta x_2$	490.7	0
(b)			
Model			
A	$y = \mu + \alpha x_1 + \beta x_2 + \varphi x_1 x_2$	356.6	38.4
B	$y = \mu + \alpha x_1 + \beta x_2$	326.5	8.3
C	$y = \mu + \alpha x_1$	318.2	0
D	$y = \mu + \beta x_2$	319.0	0.8
(c)			
Model			
A	$y = \mu + \alpha x_1 + \beta x_2 + \varphi x_1 x_2$	68.0	34.7
B	$y = \mu + \alpha x_1 + \beta x_2$	40.3	7.0
C	$y = \mu + \alpha x_1$	33.3	0.0
D	$y = \mu + \beta x_2$	34.8	1.5
(d)			
Model		AIC_c	Δ_i
A	$y = \mu + \alpha x_1 + \beta x_2 + \varphi x_1 x_2$	352.1	36.5
B	$y = \mu + \alpha x_1 + \beta x_2$	323.7	8.1
C	$y = \mu + \alpha x_1$	315.6	0
D	$y = \mu + \beta x_2$	326.1	10.5
(e)			
Model			
A	$y = \mu + \alpha x_1 + \beta x_2 + \varphi x_1 x_2$	59.8	26.3
B	$y = \mu + \alpha x_1 + \beta x_2$	39.6	6.1
C	$y = \mu + \alpha x_1$	33.5	0
D	$y = \mu + \beta x_2$	51.2	17.7

2.10 Figures

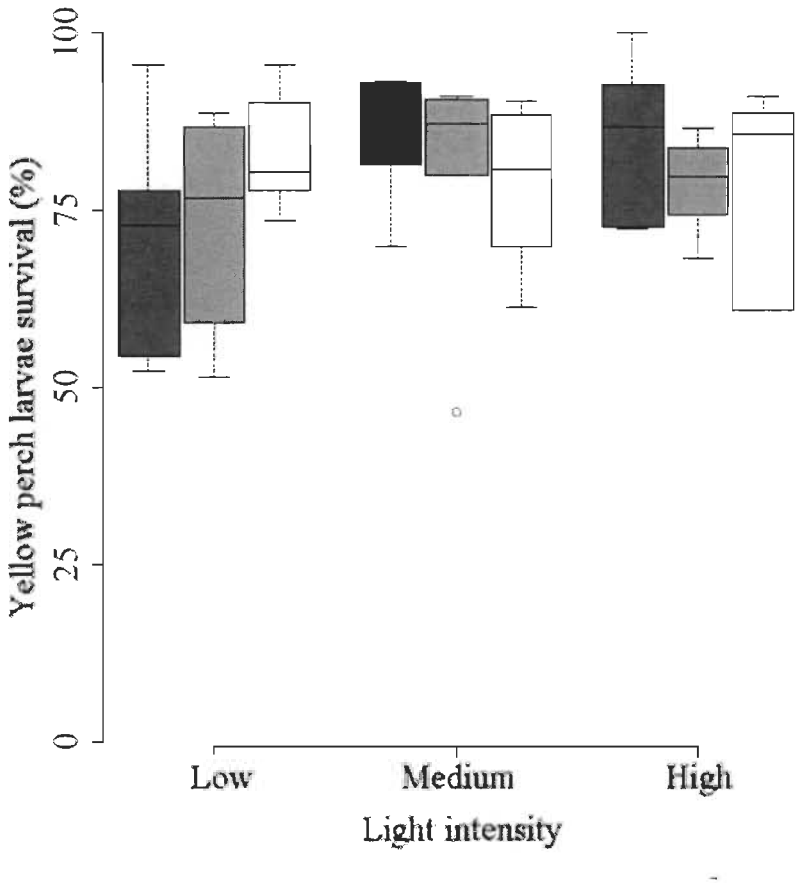

Figure 2.1 Effects of UVR quality and intensity on Yellow perch larvae survival. Whirlpak® (visible + UV-A + UV-B), *dark grey*, Mylar-*d*® (visible + UV-A), *light grey*, and J-Roll® (visible), *white*. Box plots show median values with the 25th and 75th percentiles. The bars represent the range of observed values that fall within 1.5 times the interquartile range (25-50%). Open dots represent data outside this range. None of the treatments influences larvae survival after two days of exposition.

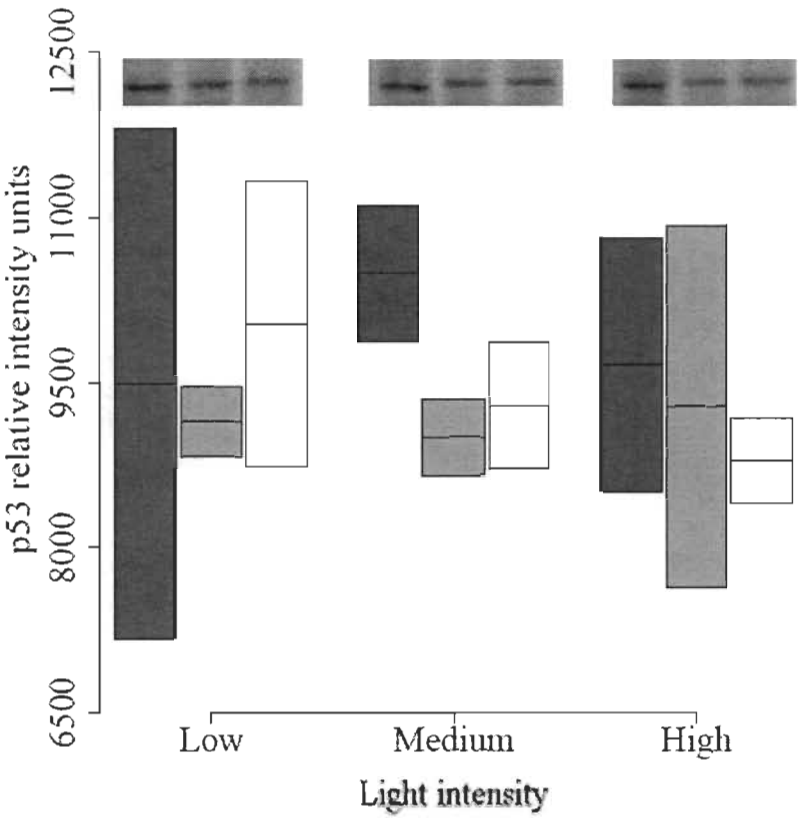

Figure 2.2 Effects of UVR quality and intensity on p53 protein relative intensity units expression. Data are shown as in Fig.2.1. None of the treatments influences p53 expression after two days of exposition

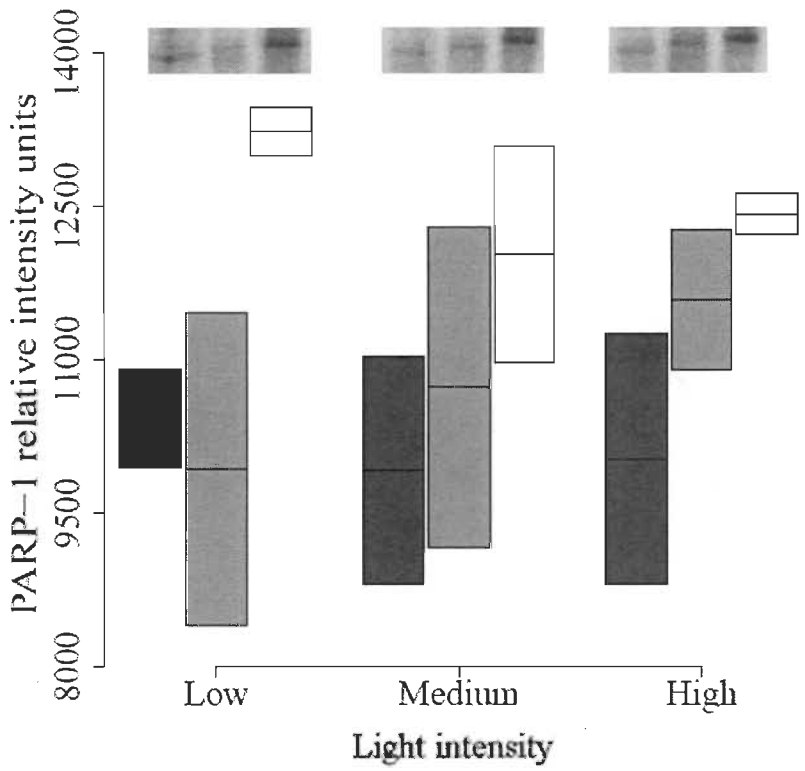

Figure 2.4 Effects of UVR quality and intensity on PARP-1 protein relative intensity units expression. Data are shown as in Fig.2.1. PARP-1 expression changes in function of light quality: treatments with UV-A+visible or UV-B+UV-A+visible reduce PARP-1 expression.

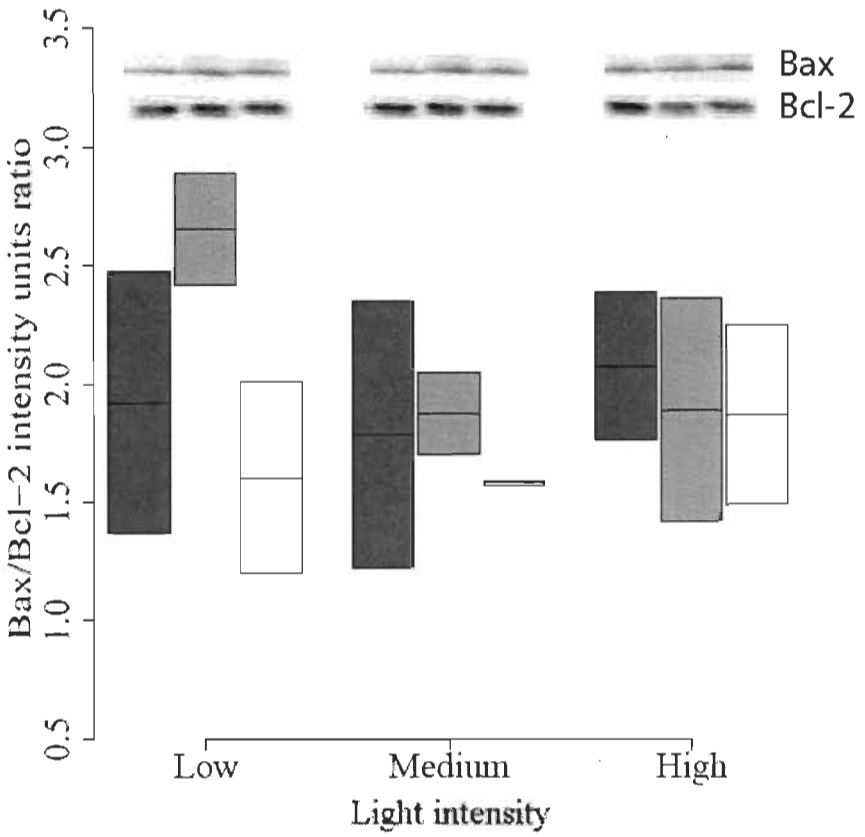

Figure 2.3 Effects of UVR quality and intensity on the Bax/Bcl-2 ratio of protein expression. Data are shown as in Fig.2.1. None of the treatments influences Bax/Bcl-2 ratio after two days of exposition.

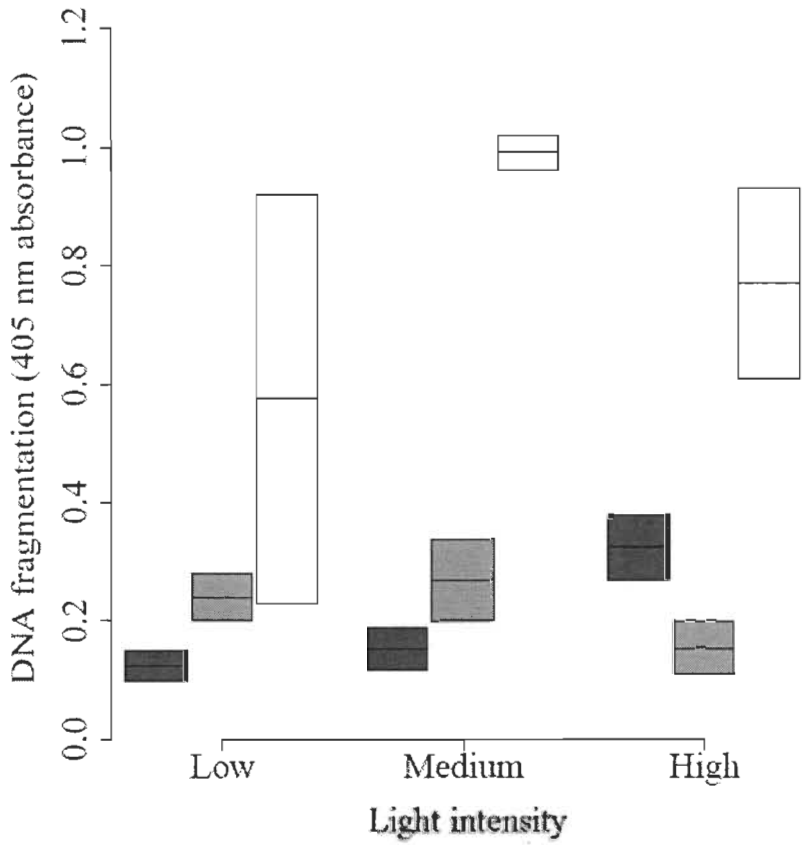

Figure 2.5 Effects of UVR quality and intensity on apoptosis. DNA fragmentation was measured in function of received light treatment. Data are shown as in Fig.2.1. DNA fragmentation changes in function of light quality: treatments with UV-A+visible or UV-B+UV-A+visible reduce DNA fragmentation.

ANNEXE 1

INSTRUCTIONS AUX AUTEURS

ENVIRONMENTAL RESEARCH

A Multidisciplinary Journal of Environmental Sciences, Ecology, and Public Health

ELSEVIER

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0013-9351

DESCRIPTION

Environmental Research: A Multidisciplinary Journal of Environmental Sciences, Ecology, and Public Health publishes original reports describing studies of the toxic effects of environmental agents on humans and animals. The principal aims of the journal are to define the etiology of environmentally induced illness and to increase understanding of the mechanisms by which environmental agents cause disease.

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INTRODUCTION

A Multidisciplinary Journal of Environmental Sciences, Ecology, and Public Health

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ANNEXE 2

CONTRIBUTION SPÉCIFIQUE DES AUTEURS

Pendant mes études de maîtrise, j'ai effectué toutes les expérimentations et recherches liées à mon projet. J'ai également fait la mise au point de la majorité des protocoles à adapter pour les larves de perchaude ayant permis d'obtenir les résultats présentés dans l'article "The effects of UVR irradiance and spectral composition on apoptosis in yellow Perch (*Perca flavescens*) larvae".

Ma contribution spécifique à cet article correspond aux manipulations ainsi qu'à tous les résultats présentés. J'ai rédigé l'article en entier, préparé les figures et rédigé leur description. La révision de l'article a été faite par le Dr Andrea Bertolo et la Dre Maria-Grazia Martinoli.

ANNEXE 3

FIGURE 1 DANS BOILY ET AL. 2011

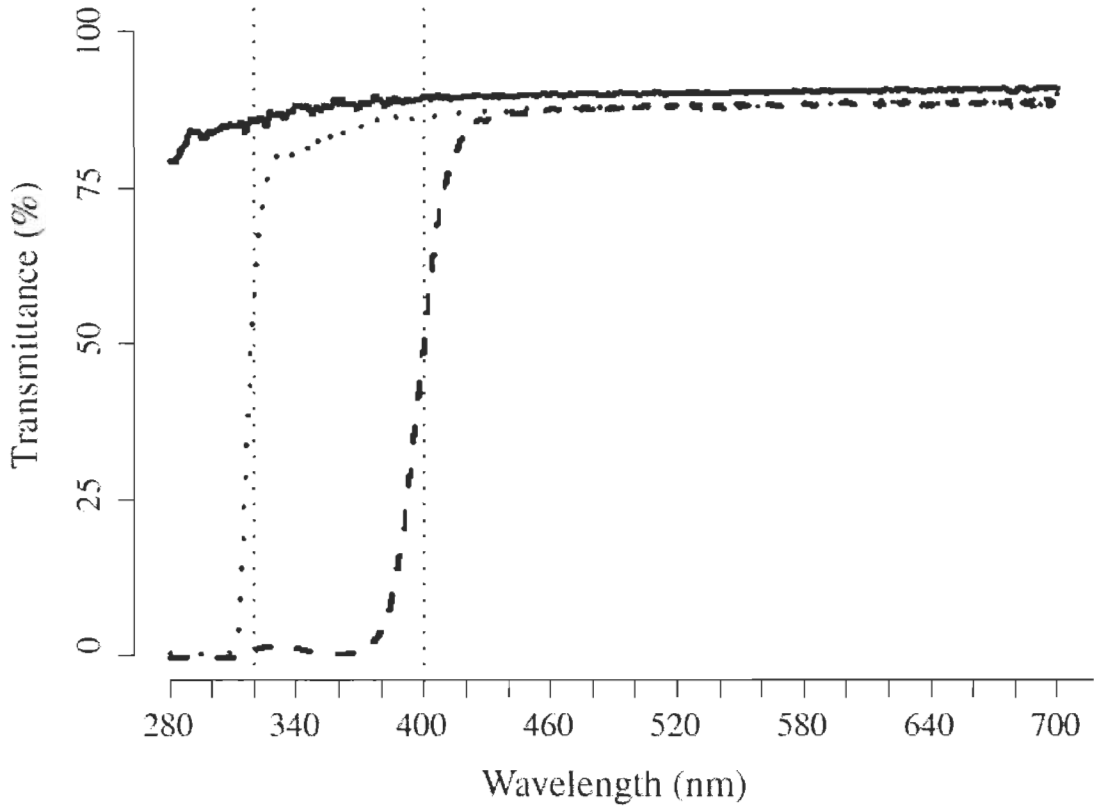

Figure 1 : Percent transmittance of filters : Whirlpak® (UV-B + UV-A + visible light) *solid line*; Mylar-*d*® (UV-A + visible light) *dotted line*; JRoll® (visible light) *dashed line*. *Vertical lines* indicate the thresholds between UV-B and UV-A (320 nm) and between UV-A and visible light (400 nm).

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